

Extraction, Encapsulation, and Microfluidization of Naringin from Grapefruit

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Abstract

Naringenin and naringin, flavonoids found in citrus fruits, may possess antioxidative, anti-inflammatory, hypolipidemic, and anticancer properties - such as mitigation of cachexia symptoms. Encapsulation can likely improve their solubility, bioavailability, and bitter taste. The usage of extracted naringin from grapefruit for potential health enhancement could decrease cost while meeting food additive regulations. Previous work in our lab has demonstrated high encapsulation efficiencies of naringin standards with β -cyclodextrin (β -CD). It was, therefore, hypothesized that under the same conditions, naringin extracted from grapefruit would also complex efficiently with β -CD. First, food-safe methods to extract naringin from a grapefruit peel powder were compared. High-performance liquid chromatography (HPLC) measured naringin concentrations, and sonication and increased extraction volume improved extraction efficiencies by 57% to 2.47 ± 0.15 g naringin /100 g grapefruit peel powder, consistent with literature results. Second, the complexation efficiencies of β -CD with extracted naringin using kneading and stirring methods were compared. Analysis by nuclear magnetic resonance spectroscopy (H-NMR) was complicated by interference from extracted impurities. One unknown peak was found in the NMR spectra for encapsulations prepared by a kneading method, which suggested a difference for this method. Lastly, model systems with naringin and naringenin were processed by microfluidization to evaluate whether high shear could convert naringin into naringenin for bioavailability improvement; results strongly suggest that it cannot. Future work to clarify encapsulation efficiency is needed, but along with previous studies, this pre-translational work can help lead to future trials in cancer cachexia mitigation, or more generally, health promotion from naringin intake.

Introduction

Naringenin and naringin, flavonoids found in many citrus fruits, offer a wide variety of health benefits due to their potential antioxidative, anti-inflammatory, and anticancer effects (Mir and Tiku 2015; Tripoli et al. 2007). During metabolism, naringin, a glycosylated flavanone, is hydrolyzed by the gut microflora and converted into naringenin, an aglycone flavone, which can then be absorbed by epithelial cells and processed by the liver before entering circulation (Mir and Tiku 2015; Shulman et al. 2011; Tripoli et al. 2007). There are several limitations in providing naringenin for intake in commercial products or in clinical trials. First, although grapefruits contain the highest concentration of naringenin of any citrus fruit, an unrealistic amount of the fruit would need to be consumed to match the relative amount of naringenin used in most cell or animal studies from which its bioactive properties have been reported (Mir and Tiku 2015; Shulman et al. 2011; Tripoli et al. 2007; Yusof et al. 1990). Second, the bioavailability of naringenin is low, and the bioavailability of naringin is even lower, limiting clinical relevance (Shulman et al. 2011). Third, the FDA has only approved naringin for GRAS status (generally recognized as safe) and not naringenin (Code of Federal Regulations 2020). Fourth, naringin is responsible for bitterness in grapefruits, which may lead to low compliance rates in a clinical trial or an

unpopular commercial product (Yusof et al. 1990). Fifth, purified forms of naringin and naringenin are relatively expensive.

A promising solution to these problems is to create a grapefruit-based product with increased naringin and/or naringenin, which would increase bioactive concentrations to levels that may be sufficient for health benefits - work towards this has been underway for several years (Niezgoda 2015; Nishikawa 2019). Additionally, encapsulation of naringenin with beta-cyclodextrin (β -CD) is likely to increase naringenin bioavailability and reduce perceived bitterness (Shulman et al. 2011; Szejtli and Sente 2005). β -CD is a cyclic oligosaccharide connected by α (1-4) glycosidic linkages, and its hydrophilic exterior and less hydrophilic interior/cavity favors formation of inclusion complexes with suitable hydrophobic molecules - such as the hydrophobic C ring of naringenin - in its interior (Crini et al. 2018; Shulman et al. 2011; Yang et al. 2013). The mechanism of bioavailability improvement by inclusion in β -CD appears to be an increase in naringenin solubility and in transport across the intestinal mucosa (Shulman et al. 2011). A reduction in bitterness is expected after encapsulation with β -CD due to its ability to block interactions of bitter compounds with taste buds (Shulman et al. 2011; Szejtli and Sente 2005).

Previous results from Nishikawa (2019) have shown that naringenin can mitigate metabolic disturbances related to cancer cachexia in a C-26 mouse model, improving muscle function, insulin sensitivity, and normalizing plasma IL-6 levels. Additionally, high encapsulation efficiencies of purified naringin and naringenin with β -CD were demonstrated using a stirring method (Nishikawa 2019). From this result, it was hypothesized that extracted naringin will also form an inclusion complex with β -CD. Encapsulation efficiencies were expected to be lower due to remaining impurities after a solvent-based extraction. - Work with characterizing and encapsulating extracted naringin would advance research in the area by satisfying regulatory demands and reducing cost. The feasibility conversion of naringin to its more bioavailable aglycone form, naringenin— using high shear was also investigated using microfluidization.

The aims of this work were threefold: to develop an efficient food-safe extraction method for grapefruit naringin, to characterize the complexation efficiencies of naringin with β -cyclodextrin for kneading and mixing methods, and to determine if microfluidization could successfully convert naringin into naringenin.

Methods

A. Naringin extraction

Flavonoids were extracted from a grapefruit peel powder (Mountain Rose Herbs, Eugene, OR) - and not juice - due to expected higher flavonoid concentrations and because, as a common waste product, its usage for future work would likely be economical and help to reduce food waste (Yusof et al. 1990). The procedures of two published solvent-based naringenin extraction methods were performed and compared, but ethanol was substituted for methanol to develop a food-safe method (Bronner and Beecher 1995; Yusof et al. 1990). Next, the method from Yusof and coworkers was optimized by addition of sonication and increased solvent volume and was used for the remainder of this work (1990). The final extraction method used was as follows: 0.100 g of grapefruit peel powder was dissolved in 10 mL of ethanol, sonicated for 30 minutes, and centrifuged at 5000 rpm for 5 minutes. Next, the supernatant was dried under nitrogen flow, and previous steps were repeated for a total of three extraction rounds.

B. Encapsulation

The efficiencies of various encapsulation techniques were compared to determine the optimal method of grapefruit flavonoid complexation with β -CD.

1. **Mixing:** Extracted naringin and β -CD were added in a 3:1 molar ratio and dissolved in a 1:4 (v/v) ethanol:water solution, with 50 mL ethanol solution added per 0.15 mmol naringin. Next, samples were filtered through a 0.45 μ m PTFE filter, sealed with Parafilm, and stirred for 5 days at room temperature. Subsequently, excess ethanol was removed by boiling, and the mixture was dried in a vacuum oven under 24 Hg pressure for 1.75 hours at 60° C, followed by 2.5 hours drying at 85° C. The dried mixture was sealed and stored at 4° C.
2. **Kneading:** In the same molar and solvent ratios used for the mixing method, dried extracts were manually kneaded into a paste for 40 minutes in a mortar and pestle. However, a 75% ethanol (aq) solution was used. Drying and storage steps followed the mixing method.
3. **Physical mixture/ control:** Similar ratios of naringin, β -CD, and 75% ethanol (aq) were combined with no additional treatment. Samples were dried and stored following the mixing method.

C. Microfluidization

High-shear processing was performed with a M-110P Microfluidizer equipped with a G10Z interaction chamber (Microfluidics International Corporation, Westwood, MA). Model systems were prepared by dissolving naringin and naringenin standards (Sigma-Aldrich, St. Louis, MO) in DI water. Naringin, naringenin, and equivalent naringin/naringenin mixtures were then processed at an operating pressure of 30,000 psi for 0-5 passes.

D. Sample analysis

4. **High-performance liquid chromatography (HPLC):** An Agilent 1100 Series (Agilent Technologies, Santa Clara, CA) was used to run a gradient elution on a reverse-phase activated C18 column for flavonoid detection and quantitation. Using 0.1% formic acid (aq) (A) and 100% acetonitrile (B), the percentage of solvent A was as follows: 100% at 0 min, 80% at 10 min, 70% at 12 min, 50% at 14 min, and 0% at 16 min. Other machine settings included an injection volume of 10 μ L, a flow rate of 1.3 mL/min, a column temperature of 25° C, and photodiode detection with a spectrum from 190 to 400 nm with signals at 283 and 288 nm. A standard curve was constructed using both naringin and naringenin standards for quantitation (Sigma-Aldrich, St. Louis, MO).
5. **Nuclear magnetic resonance spectroscopy (NMR):** 1 mg of sample was dissolved in 0.6 mL NMR-grade deuterium oxide (Sigma-Aldrich, St. Louis, MO). After transfer into a Bruker Economy NMR Tube, a ¹H-NMR spectrum was obtained at 298° K using a Bruker Avance III-HD Ultrashield 600 MHz NMR Spectrometer (Bruker Corporation, Billerica, MA). Additionally, a noesypr1d experiment was used prior to analysis for suppression of water signals.

Results

First, published extraction methods with substitution to a food safe solvent, ethanol, were compared. Naringin concentrations after extraction from a grapefruit peel powder were reported as 1.57 and 0.87 g/100g for methods described by Bronner et al. and Yusof et al., respectively (1990; 1995) (Figure 1). Ethyl acetate used for organic fractionation in the Yusof method also contained naringin, which was reported as 0.061 g/100g (Figure 1). Naringenin was not detected in any extracts. Next, the method described by Bronner and coworkers was modified as described previously; extraction efficiency was improved to 2.47 ± 0.15 g naringin /100g grapefruit peel powder (1995) (Figure 1). This result is in agreement with literature values, such those reported by Sudto et al. for pomelo peel (1.6 - 2.4 g naringin / 100g peel on a dry weight basis, depending on cultivar) and Wu et al. for fresh grapefruit peels (1.4 g naringin / 100g peel) (2009; 2007). Additional experiments were performed by modifying extraction volume to 5 mL ethanol, and no significant difference was found on naringin extraction efficiency compared to 10 mL volume (data not shown).

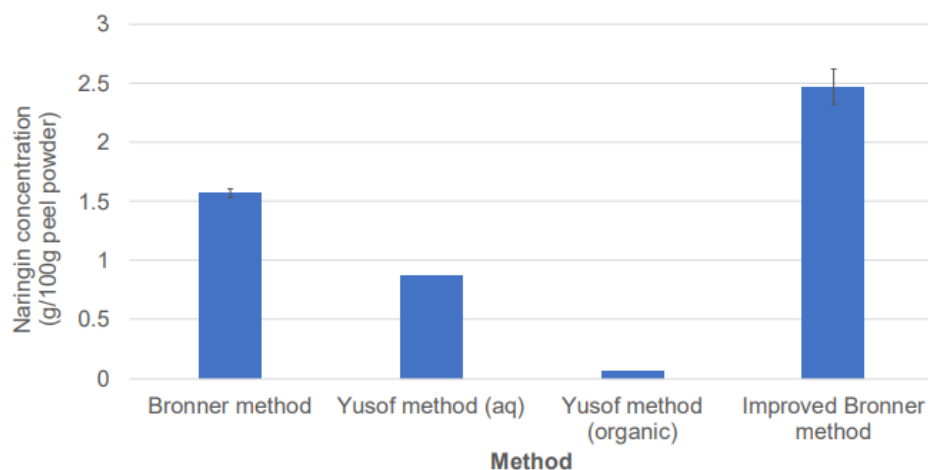


Figure 1: Comparison of methods' extraction efficiencies of naringin from grapefruit peel powder, with standard deviations provided by error bars

H-NMR spectra were obtained for analysis of encapsulation efficiencies. Compared to previous results with naringin/naringenin standards and β -CD, NMR spectra were more complex, and results were difficult to interpret (Nishikawa 2019). Spectra showed stretching of singlet peaks at 3.9 ppm in control, kneading method, and stirring method samples, which could indicate either successful inclusion complex formation of β -CD with naringin for all samples or a consistent lack of complexation (highlighted in Figure 2). Differences in compound interactions were observed between the kneading method versus the stirring and control methods based on the presence of an additional quartet peak near 3.6 ppm, but the peak remains unidentified (highlighted in Figure 2).

On a dry weight basis, more than two-thirds of grapefruit components are soluble in alcohol, so the complexity of the resulting NMR was expected. Carbohydrates are the major component of grapefruit peels, and the main alcohol-soluble sugars found in peels are glucose, fructose, and sucrose (Ting and Deszyck 1961). H-NMR spectra for glucose in D_2O have shown 6 peaks between 3.48 - 3.78 ppm, and an additional peak near 5.18 ppm, which may correspond with a peak found in control, kneading, and stirring (Figure 2) (Bagno et al. 2007). Similarly, the H-NMR spectra of fructose in D_2O contains 6 peaks between 3.56 - 4.65 ppm for all potential tautomers and may contribute to complicating the obtained spectra. The fructose tautomer α -furanose and its keto form, which both have been shown to have NMR peaks beyond 4.0, were not identified (Barclay et al. 2012). Interestingly, reported H-NMR spectra of sucrose in deuterated water contain many peaks between 4.3 - 5.2 ppm, including a prominent peak near 4.35 ppm, but this profile does not match the region of the NMR spectra in this work (Barclay et al. 2013).

Overall, these results serve as an excellent example of the often-encountered challenge faced by food scientists when moving from a model system into analysis of food products. Spectra peaks and encapsulation efficiencies could potentially be further clarified by use of an internal standard. It should be noted that another technique, differential scanning calorimetry (DSC), was also employed to characterize encapsulations but was unable to provide quality melting profiles, likely due to moisture content pressurizing sample pans upon heat treatment (data not shown).

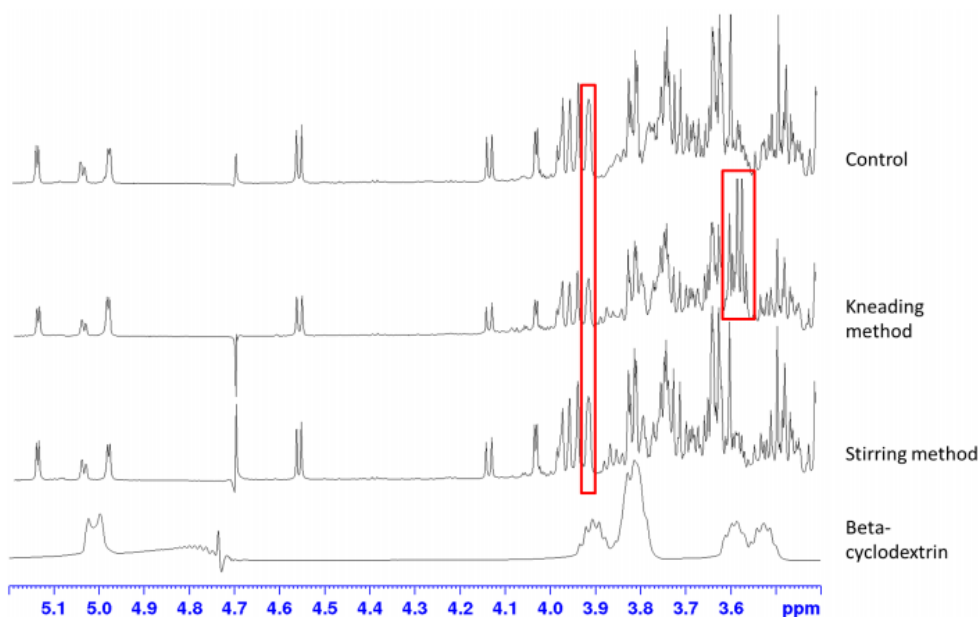


Figure 2: H-NMR spectra of naringin extracts with β -CD after various encapsulation methods and pure β -CD

Last, naringin and naringenin were shown to be stable against pressures near 30,000 psi in microfluidization processing (Figure 3). Thus, it was determined that shear alone could not convert naringin (glycosylated form) to naringenin (aglycone form) by cleavage of its glycosidic linkage. Note that for this section of the study, model systems were used to ensure that analysis was unambiguous. The outcome of naringin treatment differs from previous work with polysaccharides- namely high-methoxyl pectins, xanthan gums, and tragacanth gums - for which mechanical forces alone were sufficient to break covalent (glycosidic) bonds (Chen et al 2012; Lagoueyte and Paquin 1998; Silvestri et al. 1991). Although spectrophotometric data strongly suggested that microfluidization had no impact on naringin or naringenin, further chromatographic testing (HPLC) will be used to further validate these results.

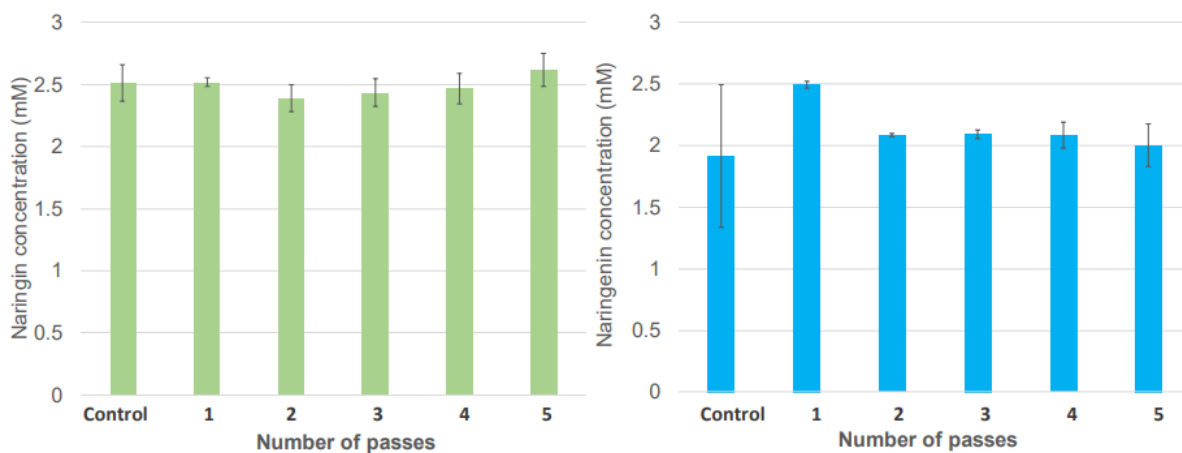


Figure 3: Naringin and naringenin concentrations in model systems after 0-5 passes through a microfluidizer, with standard deviations provided by error bars

Conclusion

A food-safe, solvent-based method for extracting naringin from grapefruit peel was developed and was aided by increasing the extraction volume of ethanol and by sonication of samples. The encapsulation efficiencies of naringin with β -CD remain unclear after H-NMR analysis, and an interesting difference was found in encapsulations prepared by a kneading method. This result showcases the difficulties of analysis beyond model systems; however, the use of an internal standard will aid in interpretation of NMR spectra for future work. As demonstrated by a model system, microfluidization alone is likely an insufficient method for conversion of naringin to naringenin. Further work is needed, but small advances made in this study together with previous publications on naringin may lead to future applications involving the nutraceutical properties of naringin to promote human health, with promising applications towards mitigating cancer cachexia.

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